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Sustained delivery of endostatin improves the efficacy of therapy in Lewis lung cancer model

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ABSTRACT

The purpose of this work was to develop an effective delivery system for antiangiogenic therapy. Endostatin was microencapsulated into poly(lactic-co-glycolic acid) (PLGA) microspheres by using a w/o/o multiple emulsification–evaporation technique. Endostatin microspheres showed the encapsulation efficiency 100% with mean particle size about 25 μm. Endostatin released *in vitro* from PLGA microspheres were biologically active and significantly inhibited the migration of endothelial cells. In rats, endostatin microspheres produced a sustained release process in which the steady-state concentration was reached from day 5 to day 27 with the steady-state levels of endostatin between 174.8±33.3 and 351.3±126.3 ng/ml. In Lewis lung cancer model, a dose of 10 mg/kg endostatin microspheres was just as effective in suppressing tumor growth as a dose of 2 mg/kg/day free endostatin for 35 days (total dose 70 mg/kg). These results indicated PLGA microspheres further reduced the amount of endostatin needed to achieve significant tumor inhibition in mice when compared with systemic administration.

1. Introduction

Antiangiogenic therapy has become a promising option for the treatment of malignant disease. Growth of solid tumors above a volume of 1 mm^3 inevitably demands the generation of new blood vessels as the nutrition and oxygen supply of proliferating tumor cells can no longer be supplied by means of diffusion [1]. Since the first report in 1997 [2], endostatin has been known to inhibit vascular endothelial cell proliferation specifically [3,4], thereby inhibiting angiogenesis and tumor growth. *In vivo* endostatin has been demonstrated to be effective in different tumor models such as Lewis lung carcinoma, T241 fibrosarcoma and B16F10 melanoma [5–11]. In 2005, the State FDA in China approved endostatin (Endostar) for the treatment of non-small-cell lung cancer. However, because angiogenesis depends on the local net result between positive and negative regulators it has been suggested that the prolonged, daily delivery of antiangiogenic therapy is the most effective way to obtain long-term suppression of tumor angiogenesis [12]. Furthermore, in order to avoid tumor recurrence, this form of therapy need to be carried out for the rest of the patient's life [2,12–14]. Therefore, it is very necessary to develop a long-term delivery system for endostatin administration.

Previous study showed that the sustained release of endostatin from osmotic pump resulted in the same antitumor efficacy as a 8–10 times higher dose administered by daily i.p. bolus injections [15]. However, these osmotic pumps cannot be injected and must be placed subcutaneously after a surgical procedure. Poly(lactic-co-glycolic acid) (PLGA) microspheres have been used as a sustained delivery system of many proteins [16–21]. It can be easily injected to any site due to their size and spherical shape. After the microspheres release their total content, freshly loaded microspheres can be injected directly without an invasive surgical procedure. Furthermore, by varying the co-monomers ratio, the time of release can be changed from weeks to months.

The most common emulsification technique for protein micro-encapsulation in PLGA microspheres is the W/O/W emulsion method [22,23]. The process begins with the use of a volatile organic solvent to dissolve the polymer. The protein aqueous solution is then dispersed in the polymer solution to form a w/o emulsion. Finally, a w/o/w double emulsion is produced by dispersing the w/o emulsion in water through mechanical mixing. Removal of the organic solvent by evaporation results in the formation of microspheres. However, because hydrophilic protein is easy to diffuse into the outer processing water, loss of protein during the preparation process is considerable. This is usually a distinct drawback in terms of efficient use of the therapeutic protein. W/O/O emulsification technique may be another choice [24]. In the method, oil is the outer processing medium [25–27] and protein cannot diffuse into the processing medium, so the total protein encapsulation efficiency is nearly 100% [28–30].

In order to develop an effective delivery system for the anti-angiogenic therapy, endostatin was microencapsulated into PLGA

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microspheres by W/O/O emulsification technique. The Lewis lung cancer model was used to determine whether the endostatin microspheres had improved the efficacy of therapy.

2. Materials and methods

2.1. Materials

Poly(D,L)-lactic-co-glycolide acid (PLGA, 0.37 dl/g, 50:50) were purchased from Birmingham Polymers, Inc.(Birmingham, AL, USA). Endostatin (trade name Endostar) was a gift from Jiangsu Simcere Pharmaceutical R&D Co., Ltd (Nanjing, China). Bicinchoninic acid (BCA) protein assay kit was purchased from Xitang Biotechnical Company (Shanghai, China). All other chemicals, including liquid paraffin, Span 80, petroleum ether (60–90 °C), acetonitrile and dimethylene chloride (DMC) were analytical grade and used as received.

2.2. Animals

Male C57B16/J mice (18–22 g) were supplied by the Model Animal Research Center of Nanjing University. Male Sprague–Dawley rats (250–300 g) were supplied from Qinglong Mountain Experimental Animal Center (Nanjing, China). Animals were acclimatized at a temperature of 25±2 °C and a relative humidity of 75±5% under natural light–dark cycle (7:00–19:00) for 1 week before dosing. The obtained food and water was available freely.

2.3. Microsphere preparation

Endostatin loaded PLGA microspheres were prepared by a modified w/o/o emulsion solvent evaporation method [24–26]. Briefly, 200 mg of PLGA was dissolved in 2 ml of acetonitrile/DMC. 10 mg of endostatin was dissolved in 0.2 ml of double-distilled water. The aqueous solution was mixed with the above organic solution and emulsified with homogenizer (XHF-1 Homogenizer, Jinda Biochemical Equipment Co., Ltd., Shanghai, China) at 4000 rpm for 30 s to form water-in-oil (w/o) emulsion. The primary w/o emulsion was then emulsified into 50 ml of liquid paraffin containing 0.3% Span80 with an electric mixer (Jintan Zhengji Equipment Co., Ltd., Jiangsu, China). The mixture was stirred at 750 rpm for 12 h in the room temperature to evaporate the organic solvents. The microspheres were collected by centrifugation at 4000 rpm, washed three times with petroleum ether. Finally, microspheres were freeze-dried and stored at 4 °C until use.

2.4. Assessment of the protein encapsulation efficiency

10 mg of the microspheres were dissolved in 1 ml of a 0.1 M NaOH/5% sodium dodecyl sulfate solution (NaOH/SDS) [31] at 37 °C for 24 h. The drug concentration in solution was estimated using BCA protein assay. Each sample was assayed in triplicate. The encapsulation efficiency was expressed by relating the actual endostatin entrapment to the theoretical endostatin entrapment.

2.5. Particle size and surface morphology

The size distribution of the microspheres was measured by Mastesizer 2000 (Malvern, Wascester, UK). The surface morphology of microspheres was analyzed by SEM (Philips XL-30 ESEM). Dried microspheres were mounted onto stubs using double-sided adhesive tape, vacuum-coated with a gold-palladium film and directly analyzed with SEM.

2.6. In vitro release experiments

The release of endostatin from the microspheres was studied as follows. 100 mg of microspheres was placed in a tube containing 10 ml of PBS and shaken with a rotary shaker at 200 rpm and 37±0.5 °C. At appropriate intervals, the samples were centrifuged at 4000 rpm for 5 min. The supernatants (1 ml) were collected and fresh buffer(1 ml) was added. The amount of protein in the supernatants was determined by ELISA (CALBIOCHEM, Inc. La Jolla, CA) according to the manufacturer's instructions. Endostatin release profiles were generated in terms of cumulative protein release versus time.

2.7. Biological activity of endostatin in an in vitro assay

Microspheres were suspended in PBS (pH 7.4) in tubes. The tubes were shaken with a rotary shaker at 37 °C. At appropriate intervals, the samples were taken and fresh buffer was added. The protein integrity of endostatin was analyzed by using a Shimadzu HPLC system equipped with a Bio-Gel SEC 50-XL size-exclusion column (BIO-RAD, USA). PBS buffer (pH 7.4) was used as the mobile phase running at the flow-rate of 1.2 ml/min. Absorbance of endostatin was recorded at 214 nm. The ability of endostatin to inhibit the migration of endothelial cells was assayed as described below [15]. Human umbilical vein endothelial cells, passage 4, were maintained in Medium 199 (Gibco, Grand Island, NY, USA), 20% fetal bovine serum (Gibco), endothelial cell growth supplement (ECGS, 30 μg/ml, Sigma), epidermal growth factor (EGF 10 ng/ml, Sigma), 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were trypsinized, centrifuged, and diluted in Medium 199 with 0.05% gelatin. Cells were preincubated for 30 min with or without endostatin at a concentration of 500 ng/ml at 37 °C. Cells (1.5×10^5) in 300 μl were added per well to 10-mm tissue culture inserts (Millicell, Billerico, USA; 8um pore) that had been treated with 10 μg/ml of fibronectin. Medium (300 μl) was added to the bottom wells with 10 ng/ml of VEGF (Sigma), and cells were incubated for 9 h at 37 °C. Cells were washed once with PBS, and the cells that had not migrated were removed from the top membrane by scraping with a cotton swab. Cells that had migrated were quantitated using a colorimetric assay as follows. Cells bound to the bottom of the tissue culture inserts were incubated for 2 h in 400 μl of acid phosphatase substrate [10 mM *p*-nitrophenyl phosphate, 10 mM sodium acetate, 0.1% Triton X-100 (pH 5.8)] at 37 °C. Reaction was then quenched with 100 μl of 1 N NaOH, and absorbance of the solution was read at 410 nm.

2.8. In vivo experiment

2.8.1. Determination of the pharmacokinetics of endostatin

In vivo evaluation was performed in male Sprague–Dawley rats. The animal care and handling were performed in accordance with the guidelines and approval of the local Institutional Animal Experimentation Ethics Committee. Microspheres were suspended in dispersed medium(0.5% CMC-Na, 5% mannitol and 0.1% Tween 80) and administrated subcutaneously to the neck(the dose of peptide was 10 mg/kg). Blood samples were collected from retro-orbital plexus at different time intervals for 30 days. Endostatin concentration in blood samples was determined by ELISA (CALBIOCHEM, USA) according to the manufacturer's instructions. Data were presented as mean± standard deviation.

2.8.2. ELISA assay of anti-endostatin antibody

Anti-endostatin antibodies were determined by human anti-endostatin antibody ELISA Kit (Cusabio Biotech Co., USA) according to the manufacturer's instructions. Blood samples were collected from retro-orbital plexus prior to study initiation and on study days 30. Each serum sample was tested at dilutions of 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, 1:256, 1:512 and 1:1024. Antibody titer was defined as the dilution rate when the OD for a diluted sample approached to a cut-off value. In the present study, the cut-off OD was the OD of negative control plus 3 standard deviations (i.e. $OD > OD_{control} + 3SD_{control}$).

2.8.3. Treatment of tumor-bearing mice

Male C57BL/6 mice were subcutaneously injected with Lewis lung cancer cells in a volume of 0.2 ml (2×10^7/ml). After 24 h of tumor inoculation, mice were at random divided into 5 groups. The first group of mice was control and received dispersed medium. The second group of mice received free endostatin in dispersed medium at a dose of 2 mg/kg/day subcutaneously. The third, fourth and fifth group of mice received endostatin microspheres in dispersed medium by subcutaneous injection to the neck at the dose of 60 mg/kg, 30 mg/kg and 10 mg/kg, respectively. The mice were weighed and tumors were measured every 3–5 days in two diameters with a dial-caliper. Volumes were determined using the formula $a^2\times b\times0.52$ (where a is the shortest and b is the longest diameter). At the end of each experiment, the mice were sacrificed with methoxyflurane and the tumors were fixed in 10% formalin.

2.8.4. Immunohistochemistry

Tissues were paraffin embedded. Sections (5 μm) were first stained with H&E to evaluate tissue viability and quality. The microvessel density was determined by immunohistochemical staining using an avidin–biotin detection system (Boster, Wuhan, China) with anti-CD31 antibody (monoclonal, dilution:1:250; Biolegend, San Diego, CA) according to the manufacturer's instruction. Microvessel density was determined by image analyses software using 10 randomly chosen fields per section in at least five sections at 200×.

2.9. Statistics

All results were presented as means±SD. Statistical analysis was performed by applying the Student's t-test.

3. Results

3.1. Preparation of microspheres

As shown in Table 1, acetonitrile alone could led to endostatin precipitation. Incorporation of DCM in the solvent mixture would decrease the protein precipitation. However, when the DCM proportion was increased to 70%, the particle sizes became very large. If the DCM proportion was increased further, PLGA lump would form. It was, thus, concluded that a mixed solvent system comprising 1:1 proportion of acetonitrile and DCM yielded the desired endostatin particles. In this study, all endostatin microspheres were prepared in the proportion of 1:1.

3.2. Microsphere characterization

The external surface morphology of the PLGA microspheres prepared by W/O/O was analyzed by scanning electron microscopy. The micrographs exhibited a spherical shape with smooth and uniform surface morphology (Fig. 1). There were no pores on the smooth surface of microspheres. The microspheres size was measured by using Malvern counter. The mean particle size of endostatin microspheres was about 25 μm. The loading of endostatin in the microspheres was 10% (w/w) and the encapsulation efficiency was 100%.

Table 1
Influence of solvent composition on the microencapsulation process and product characteristics

Acetonitrile:DMC	Property of w/o_1 emulsion	Product characteristics
100:0	Protein precipitated	Very fine particles
90:10	Protein precipitated	Very fine particles
70:30	Precipitation tendency very high	Very fine particles
50:50	Homogenous	Spherical; high entrapment efficiency
30:70	Homogenous	Large particles
10:90	Homogenous	Lumps formed
0:100	Homogenous	Lumps formed

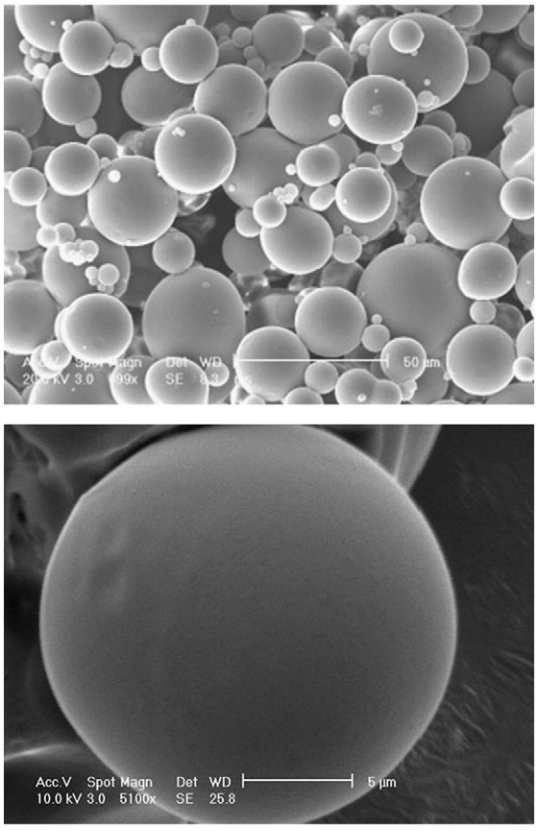

Fig. 1. Scanning electron microscopy (SEM) images of PLGA microspheres containing endostatin.

3.3. In vitro drug release studies

In this study, the release profile of endostatin from the PLGA microspheres was determined by cumulative percentage of protein release (Fig. 2). The release profile of endostatin was characterized by a controlled release of the protein over a 28-day period. However, no burst release was found in the release profile. After 28 days, the cumulative release profile got to a plateau. From day 28 to day 40, the cumulative release remained about 41%.

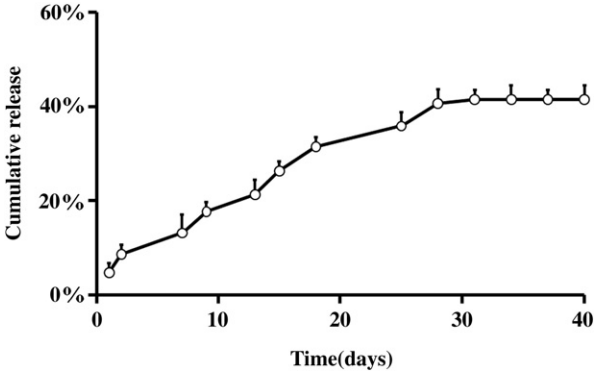

Fig. 2. Endostatin release profiles from PLGA microspheres(n=4). 100 mg of endostatin microspheres containing 10 mg endostatin were incubated in PBS at 37 °C and 200 rpm shaking.

3.4. Biological activity of endostatin in an in vitro assay

Endostatin released from microspheres was assayed by SEC-HPLC to determine any degraded or aggregated proteins. The SEC-HPLC patterns of endostatin original solution and released endostatin samples were identical (Fig. 3). The ability of endostatin to inhibit the migration of endostatin cells was also determined. As shown in Fig. 4, there was no significant difference between fresh endostatin and released samples. Clearly, the process for forming endostatin microspheres did not affect the biological activity of endostatin.

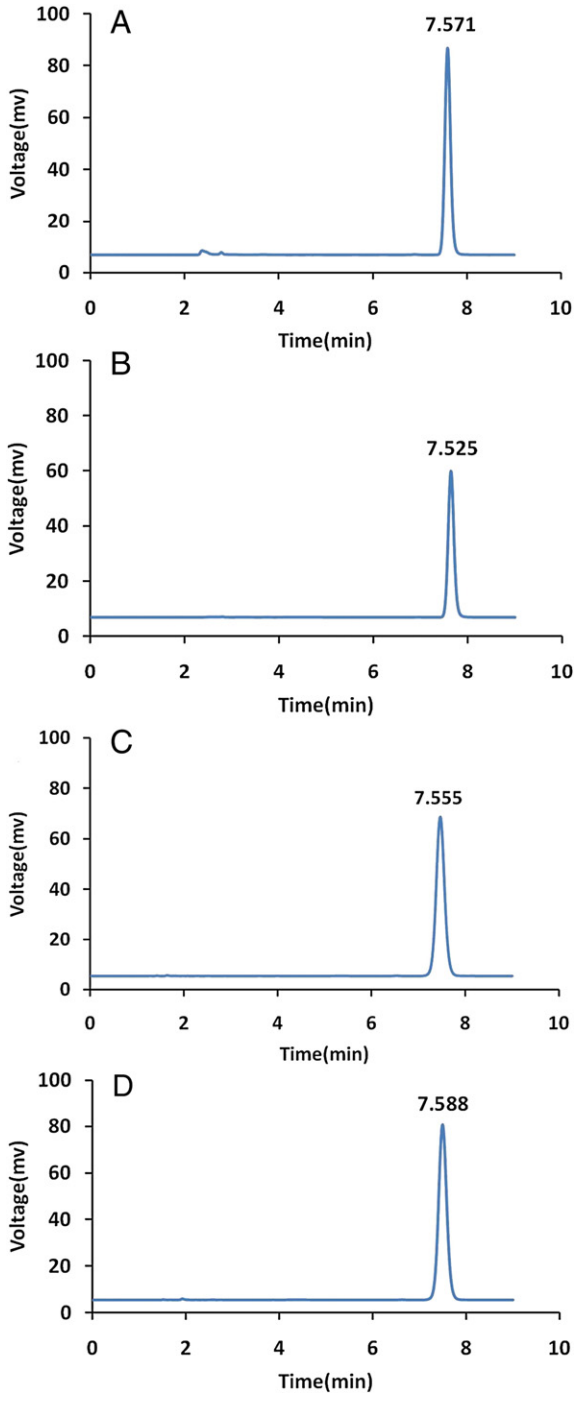

Fig. 3. SEC-HPLC patterns of endostatin. A: Fresh endostatin solution; B, C and D: released endostatin from microspheres at study days 7, 14 and 27, respectively. There is no aggregation or degradation in the release samples.

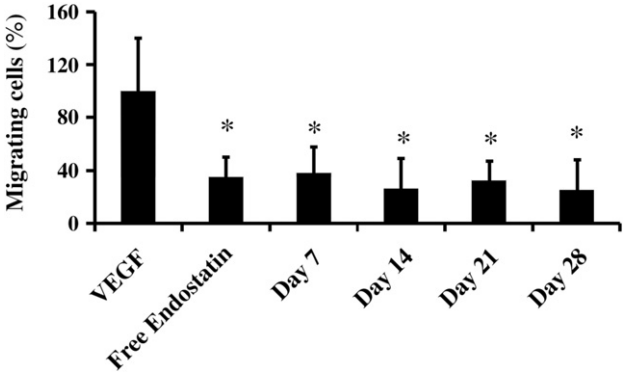

Fig. 4. Biological activity of endostatin released from microspheres at different release times ($n=3$). The full biological activity of these samples is still present after the 28 days' release. $^{*}p<0.05$, significantly different from the VEGF control.

3.5. In vivo experiment

3.5.1. Endostatin level in rat plasma

The experimental results demonstrated that endostatin microspheres produced a sustained release process (30 days) in which the steady-state concentration was reached at day 5 and maintained until day 27. The steady-state concentration levels of endostatin in rat plasma changed between 174.8±33.3 and 351.3±126.3 ng/ml with a small peak-valley situation (Fig. 5). This result offered us satisfactory experimental evidence that endostatin microspheres showed a sustained release in rats.

3.5.2. Antibody developed in rats

The anti-endostatin antibodies were not detectable in the pretreatment serum. Only one of seven rats developed low titer antibodies (1:16) on study day 30. These results demonstrated that the drop of endostatin level was due to the complete release of endostatin.

3.5.3. Treatment of tumor-bearing mice

In the present study, we evaluated the effect of endostatin microspheres on the growth of inoculated Lewis tumor in mice. After subcutaneous administration, endostatin microspheres (60 mg/kg) suppressed the growth of Lewis lung carcinoma by 80% (Fig. 6; $p<0.05$, *vs* control group) for 35 days. In addition, there was no obvious difference in tumor volume between endostatin microspheres group (10 mg/kg) and free endostatin group ($p>0.05$, Fig. 6). It was showed that endostatin microspheres (10 mg/kg) by subcutaneous injection on the back of each animal could reach the same effect as free endostatin for 35 days (total dose 70 mg/kg).

Microvessel density in histological sections of tumors was analyzed using an antibody directed against the endothelial cell marker CD31.

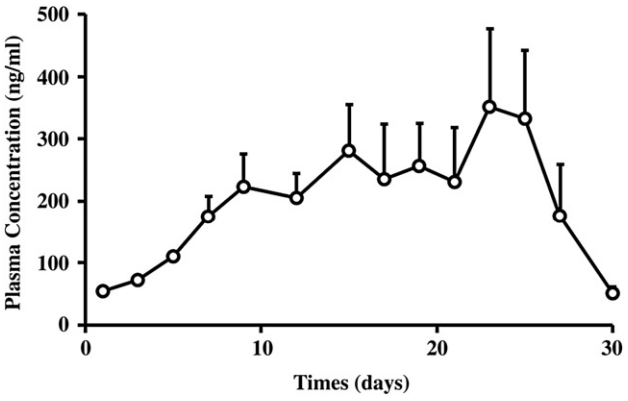

Fig. 5. Endostatin concentration curve in plasma in rats ($n=7$).

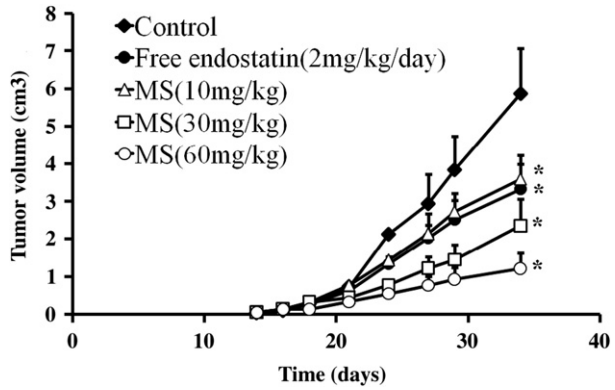

Fig. 6. Treatment of Lewis lung cancer with endostatin($n=10$). Lewis lung cancer cells were implanted in mice as described. Animals received endostatin microspheres(MS) and free endostatin. Control animals received dispersed medium. *$p<0.05$, significantly different from the control.

There was a significant difference in the microvessel density between the treatment groups and the control group (Fig. 7). Quantification of microvessel density revealed 250±65 capillaries per field in controlled group. Animals treated with free endostatin had a tumor microvessel density 160±45 capillaries per field. Animals treated with endostatin microspheres (60 mg/kg) had a tumor microvessel density of 24±22 capillaries per field. A decrease in microvessel density during treatment with endostatin microspheres suggested that the long-term delivery system was effective.

4. Discussion

In the present studies, endostatin was encapsulated into PLGA microspheres by W/O/O emulsification technique for the first time. Because the outer processing medium was oil, endostatin could not diffuse into the processing medium and high encapsulation efficiency (100%) was achieved.

With oil as the processing medium, use of acetonitrile alone as a solvent did not ensure the formation of endostatin microspheres. Immediately on mixing, the water-miscibility of acetonitrile would bring about the precipitation of protein. Hence, a small proportion of a nonpolar solvent, namely, dichloromethane was included with acetonitrile to decrease polarity of the polymer solution. Additionally, it was also desirable that the second solvent was oil-miscible so that solvent removal was facilitated by extraction by processing medium. Inclusion of an oil-extractable solvent would bring about rapid extraction immediately on introduction into processing medium [24]. But the appropriate ratio of acetonitrile to dichloromethane was very important for obtaining desired particles. If the ratio was lower than 30%, the particle size became very large due to high viscosities of polymer solution after the dichloromethane was extracted off. On the other hand, if the proportion was higher than 70%, water-miscible acetonitrile would cause the precipitation of endostatin.

As shown in the SEM images, the surface of endostatin microspheres were smooth without pores. It was expected that the evaporation rate of inner aqueous phase would determine the microsphere morphology [29]. Immediately on introduction of the water-in-oil emulsion into liquid paraffin, there was breakdown of the water-in-oil emulsion into tiny droplets and rapid extraction of dichloromethane. After all the dichloromethane was extracted off, the mixed solvent system became one component system, i.e., it comprises only acetronitrile, which was water-miscible. Simultaneously, the mixture of water and acetonitrile became a poor solvent for the polymer and was forced out of the droplet. The migration of water-acetronitrile mixture out of the droplet occurred through pores in the viscous half-formed microspheres [29]. The polymer underwent phase inversion leading to a microporous structure [24,29]. Since the microspheres possessed tendency to agglomerate until all water-acetronitrile was removed, the fluidity imparted to the polymer matrix by the water-acetonitrile mixture enabled sealing-off the pores. The sealing-off of the pores depended upon the rate of removal of acetronitrile by evaporation. A rapid evaporation under low pressure or high temperature would result in pores remaining unsealed. Slow evaporation of acetronitrile allowed sufficient time for the viscous sphere to seal-off the pores and yielded a dense internal morphology [24–26]. In the present study, the evaporation of acetronitrile was under normal temperature which led to a slow evaporation and a smooth surface.

The PLGA preparation procedure involved the use of organic solvents due to the nature of polymers and might affect the biological activity of the loaded protein. Therefore, we did *in vitro* release studies of endostatin from the PLGA microspheres and confirmed their biological activity. Results showed that endostatin released from the PLGA were able to inhibit endothelial cell migration greatly. There was no significant difference between fresh endostatin and released endostatin. Furthermore, the SEC-HPLC also showed no aggregation or degradation in the released samples demonstrating that the PLGA formulation procedure did not affect the biological activity of endostatin.

The *in vitro* release profile of endostatin was characterized by a controlled release of the protein over a 28-day period. However, no burst release was observed. It could be due to the w/o/o emulsification technique. In the method, oil was chosen as the processing medium [25–27], and protein was not easily adsorbed on the surface of microparticles due to its hydrophilicity, so the initial burst release was very low [28–30]. Endostatin microspheres also showed an incomplete release. An explanation might be the possible ionic interaction between the endostatin (positively charged amino groups) and the PLGA carboxyl-end groups (negatively charged). The isoelectric point of endostatin was 9.8 [4]. In pH 7.4 PBS, positive charged endostatin had high interaction with PLGA, which could hinder the release of protein. The same effect was also reported for other proteins [32].

Pharmacokinetic study showed that the endostatin concentration level decreased to the basal level on day 30. Because anti-endostatin antibodies were not detectable in six of seven rats on study day 30, the drop of endostatin level was due to the complete release of endostatin. Endostatin microspheres released faster *in vivo* than *in vitro,* which was due to the foreign body response [33,34]. The *in vivo* system was far more complex because of the presence of proteolytic enzymes, cellular infiltrates, various cytokines, and pH gradients [35]. The slow *in vitro* release has also been reported for many PLGA-based release systems [35–37].

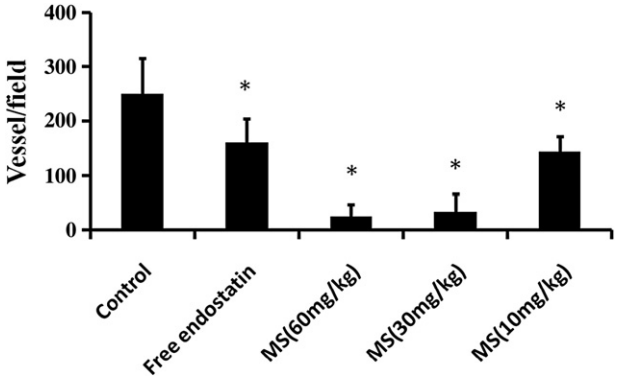

Fig. 7. Histological analysis of excised tumors. Excised tumors were fixed and stained for the presence of the endothelial cell marker CD31. Sections were then quantified for microvessel density. *$p<0.05$, significantly different from the control.

It was well documented that antiangiogenic therapy required daily administration to achieve tumor inhibition. A daily s.c. administration of 100 mg/kg endostatin for 23 days was needed to achieve 75% inhibition of Lewis lung carcinoma [15]. However, when administered by sustained delivery system, the amount of endostatin used to achieve the same tumor inhibition was reduced significantly [15]. In this study, endostatin was encapsulated into PLGA microspheres. The use of PLGA microspheres also further reduced the amount of endostatin needed to achieve significant tumor inhibition in mice when compared with systemic administration. Only a single s.c. injection of endostatin microspheres (10 mg/kg) could inhibit tumor growth by 40% for 35 days. A dose of 10 mg/kg endostatin microspheres was just as effective in suppressing tumor growth as a dose of 2 mg/kg/day free endostatin for 35 days (total dose 70 mg/kg). These data were consistent with the previous study which had showed that the continuous administration of endostatin would be more effective against tumors [15]. These could be explained by the U-shaped dose response curve of endostatin [38]. For example, at high endostatin serum levels of 15–20 μg/ml, no tumor inhibition was observed in T241 fibrosarcoma [39]. Similarly, Pawliuk et al. reported that systemic endostatin levels of 750 ng/ml also failed to inhibit T241 fibrosarcoma [40]. The U-shaped response might come from receptor desensitization (such as integrins). Also, it was reported that sustained high levels of endostatin elicited toxicity and immunogenicity. Antibody were detected in the serum and tumor tissues of a patient with multifocal glioblastoma when given at high doses, which reduced the effectiveness of endostatin and caused the U-shaped response [38,41].

5. Conclusion

The presented data showed the advantage of PLGA microspheres as a long term delivery system of angiogenic inhibitors for the treatment of cancer. The PLGA microspheres allowed the sustained release of biological active endostatin for 30 days. The use of PLGA microspheres further reduced the amount of endostatin needed to achieve significant tumor inhibition in mice when compared with systemic administration. Thus, it may be more efficacious to treat patients with endostatin microspheres.

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